April 2009

Reduced Cognition and Increased Anxiety Due to Knockdown of the Mental Retardation Gene Jarid1c in the Mouse Brain

Kelsey K. Graham
Worcester Polytechnic Institute

Project Number: DVG-KKGR

Reduced Cognition and Increased Anxiety Due to Knockdown of the Mental Retardation Gene Jarid1c in the Mouse Brain

A Major Qualifying Project Report:

Submitted to the Faculty

of

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

_____

Kelsey K Graham

Date: April 30, 2009

Approved:

_____

Daniel G. Gibson III, Ph.D., Advisor

Abstract

The gene Jarid1c plays an important role in brain function. Human mutations in Jarid1c cause mental retardation and autistic behaviors. This study looks into the importance of Jarid1c in specific brain regions speculated to be involved in autism. Knockdown of Jarid1c in BL/6 mice was performed using stereotaxic injection of Jarid1c-siRNA into the hippocampus, amygdala, and cortex. Controls were given a non-targeting siRNA. Behavior testing was performed two days post-surgery and microarray analysis was performed to confirm knockdown. It was found that Jarid1c knockdown in the hippocampus significantly reduces the mouse's ability to perform well on an object recognition test. It was also found that Jarid1c knockdown in the prefrontal cortex significantly increases the mouse's anxiety.

Table of Contents

Table of Figures

Acknowledgments

I would like to thank Dr. Jun Xu for all of his support, both with the equipment and for sharing his knowledge. Additionally, I would like to thank Dr. Liz Byrnes and Marc Siegel for their active involvement in this project as well as Alex Newbury for his skills with behavior testing. Finally, I would like to thank Dr. Dan Gibson for lending support from WPI throughout this project. This project was funded by the National Science Foundation and Tufts University internal grants awarded to Dr. Jun Xu.

Background

Jarid1c and its link to mental retardation

Mental retardation is a non-progressive cognitive impairment that affects 2-3% of the population (Chelly, 1999). Yet, even given the high frequency this disease appears in the population, research has largely neglected to find the causes of this disease. It has been discovered, however, that many genes connected to mental retardation are found on the X chromosome. This led to the discovery of a number of types of retardation now generally termed X-linked mental retardation (XLMR) (Chelly, 1999).

X-linked mental retardation is a heterogeneous disease affecting 2 out of every 1000 males. Many genes have been discovered to have a link to XLMR; one of the more recently discovered genes is Jarid1c. While there have been several other genes identified as possible causes of XLMR, Jarid1c has shown to be one of the most frequently mutated causal genes in this disease (Iwase *et al*., 2007). In humans, mutations in the Jarid1c gene cause neurological disorders including mental retardation, epilepsy, aggression, and autism.

In a study done in Australia in 2004, families with nonsyndromic X-linked mental retardation were studied and Jarid1c was discovered as a gene of interest in human brain function. Seven mutations were found in the gene among the patients studied and mutations in Jarid1c were found in 2 of 31 families investigated (Jensen *et al*., 2005). Through further investigation it was discovered that Jarid1c encodes a number of proteins involved in transcriptional regulation and chromatin remodeling. In the study, Northern blot analysis was performed for 6 affected males as well as 5 male controls. Two out of six of the affected males tested showed a significant reduction of Jarid1c transcripts, which would likely result in a non-

functional Jarid1c protein. Given the results of this study, it is now believed that the Jarid1c mutations detected significantly affect the function of the protein and are responsible for the cognitive deficits associated with the patients in this study (Jensen *et al*., 2005).

Jensen *et al*. (2005) also discovered that Jarid1c in humans is evolutionarily conserved with homologs in the mouse. This makes the mouse model an excellent tool for further research, since the Jarid1c gene plays the same role in mice as it does in the human brain. One interesting difference, however, is that in mice, Jarid1c is expressed at significantly higher levels in the adult female than in the male. Given this discrepancy between female and male mice, it would make a knockdown of Jarid1c in a male mouse brain hold more significance than in a female mouse brain. A male mouse would be a better model because there would be less compensation of the lost Jarid1c function elsewhere in the brain. For example, 25% knockdown in a male mouse would be more significant than 25% knockdown in a female since the male started off with a lower Jarid1c function level (Jensen *et al*., 2005).

Jarid1c has proven to be important in other species as well. In a 2007 study by Iwase *et al*., the influence of Jarid1c on neuronal survival and dendrite development was studied using zebrafish embryos and mammalian cells. Results indicated that Jarid1c was present throughout the fish's embryogenesis, with ubiquitous expression in early stages and then expression isolated to the head in later stages. These results suggest that Jarid1c plays a valuable role in neuronal development and survival in the zebrafish (Iwase *et al*., 2007). In this study Jarid1c function was also observed in mammalian neurons. Granule neurons from rodent cerebellar cortex were used for this study. Gene knockdown was performed using plasmid-based RNAi in cells cultured from a postnatal day six rat. It was found that knockdown led to a significant reduction in dendritic length of neurons. This suggests that Jarid1c plays an important role in dendritic development in

the rodent brain (Iwase *et al*., 2007). Given these findings, Jarid1c may play a significant role in neuronal development in the human brain as well, since the gene functions similarly in both species.

In the same study (Iwase *et al*., 2007), Jarid1c was discovered to be responsible for catalyzing demethylation of certain histones. In vitro, over expression of Jarid1c was tested and it was found to result in specific reduction of histones, in support of previous findings. The missense mutations in Jarid1c that were previously found to be related to XLMR were shown to specifically affect the genes' demethylation ability as well as its ability to bind the histones. This suggests that Jarid1c's demethylation ability is pertinent to understanding the causes of XLMR. To further test this idea, Jarid1c was inhibited by in situ hybridization in zebrafish. Significant neuronal defects were found as well as increased neuronal cell death in zebrafish with inhibited Jarid1c (Iwase *et al*., 2007).

siRNA

Small interfering RNAs (siRNAs) are used to transiently silence a target gene. They mimic intermediates in the RNAi pathway, suppress the targeted genes' expression, and do not influence expression of non-targeted genes (McCaffrey *et al*., 2002). Specifically, siRNA cleaves cytoplasmic mRNAs containing sequences that are homologous to that specific siRNAs double stranded RNA (dsRNA) trigger as shown in Figure 1 (Thakker *et al*., 2004).

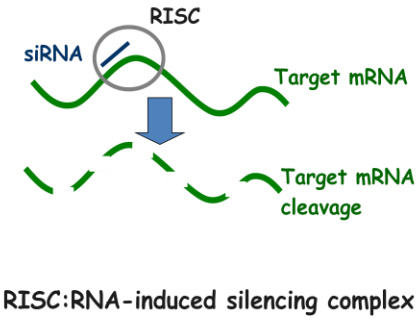

Figure 1: RNA-induced silencing complex

McCaffrey *et al*. (2002) studied the ability of siRNA to work in vivo. They tested adult rats by injecting firefly luciferase into their liver along with luciferase targeting siRNA and an unrelated siRNA as a control. The ability of the luciferase siRNA to down regulate gene expression was then monitored using the luciferase reporter (Figure 2). Images were taken 72 hours after injection and results showed significant decrease in luciferase in the mice injected with the luciferase siRNA. The controls showed no decrease in luciferase confirming that the siRNA specific to luciferase was responsible for the reduction (McCaffrey *et al*., 2002).

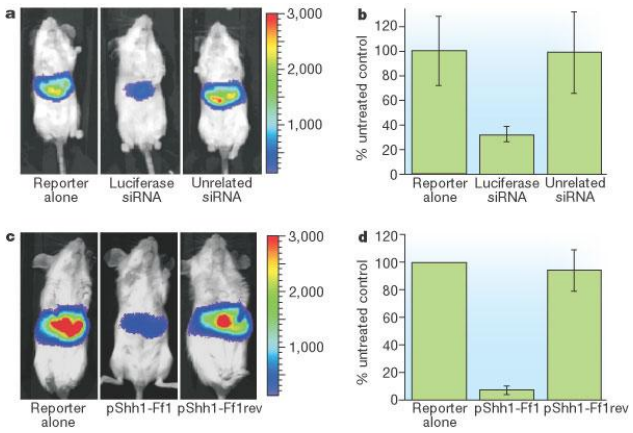

Figure 2: RNA interference in adult mice at 72 hours post-injection (McCaffrey *et al*., 2002)

Once siRNA's ability to knockdown specific genes in vivo was confirmed, other studies confirmed its use in the mammalian brain. In a 2004 study, gene specific siRNA was injected into different areas of the mouse brain and tested. The tissue was then analyzed to see if the target mRNA knockdown was successful. It was found that siRNA was successful in producing a specific and time-dependent reduction of the target gene's expression in vivo (Thakker *et al*., 2004). This study confirmed that siRNA could successfully be used in the mammalian brain for specific gene silencing.

Stereotaxic injection

Stereotaxic injection is an invaluable tool that is quickly becoming more popular for gene manipulation in the neuroscience field. There are many benefits of stereotaxic injection over traditional gene manipulation methods. The use of stereotaxic injection allows for site specific gene knockdown in combination with a targeted siRNA. It also allows for targeting of any brain region, which was much more difficult before the use of stereotaxic injection. Furthermore, knockdown spreading to other non-targeted regions of the brain is minimized when using proper stereotaxic procedure. The main benefit of stereotaxic injection is the exceptional spatiotemporal control. Exact coordinates for the target brain region can be established using a mouse atlas such as the atlas created by Paxinos & Franklin (2001). This procedure insures that the correct area is being targeted and avoids variables such as gene alterations which occur when using more traditional mouse genetics (Cetin *et al*., 2007).

Brain regions

Different brain regions have been targeted for siRNA gene knockdown in previous studies. In one study (Thakker *et al*., 2004), an enhanced green fluorescent protein gene (EGFP) was targeted and EGFP-siRNA was injected into the dorsal third ventricle to allow for bilateral and widespread distribution of the target siRNA in the mouse brain. siRNA was infused for one week and then 17 brain regions were tested for knockdown success. As shown in Figure 3, down-regulation of EGFP mRNA was successful in 11 of 17 brain regions tested. The highest knockdown was recorded in those areas closest to the injection point.

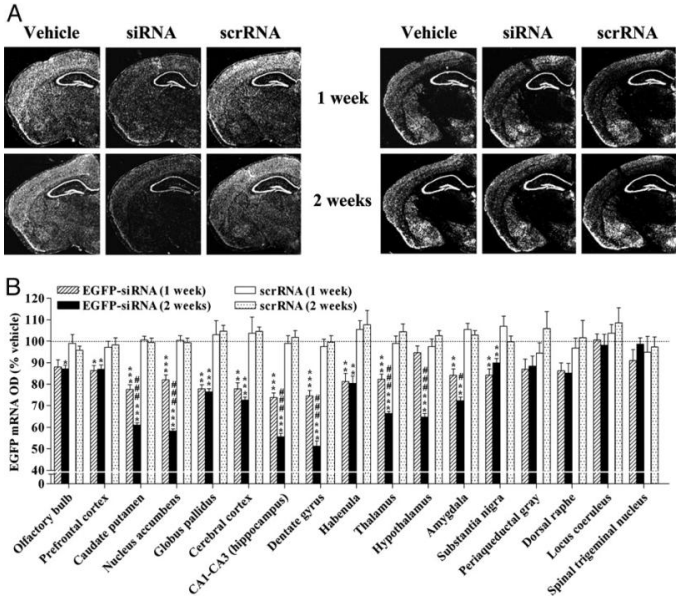

Figure 3: siRNA-induced specific, temporal, and widespread knockdown of EGFP mRNA in the brain (Thakker *et al*., 2004)

In another test, knockdown of the gene GluR2 and its protein, Cox-1, was performed in the rat brain using electroporation to deliver gene specific siRNA to certain brain regions (Akaneya *et al*., 2005). The visual cortex was targeted in one test and the hippocampus in

another. In both cases there was successful gene and protein knockdown and knockdown was specific to the targeted brain region. This study was very important in that it showed that knockdown spreading could be avoided using proper stereotaxic injection techniques.

The amygdala, prefrontal cortex and hippocampus are all associated with behaviors common to children suffering from autism or other similar XLMR diseases. The amygdala is part of what is known as the "social brain" and therefore is often the target of autistic research. In a study (Baron-Cohen *et al*., 2000) that used magnetic resonance imaging (MRI) it was found that the amygdala is not activated in children with autism when making inferences from the eyes. However, in people without autism, MRI showed significant amygdala activity during the same tests. This study shows promise for furthering autism research and focusing on the importance of the amygdala.

The prefrontal cortex has also been found to play a role in autistic behavior (Ohnishi *et al*., 2000). The prefrontal cortex is important for communication as well as social interaction. In a study involving neuroimaging of autistic patients, it was found that patients suffering from autism had altered perfusion in the medial prefrontal cortex when compared to control patients. These alterations suggest possible abnormalities in brain function that may cause the behaviors associated with autistic patients. The alterations in perfusion were especially apparent in those patients suffering from behavior such as deficits in theory of mind, abnormal sensory responses, and an obsession for sameness.

The hippocampus is important for long term memory as well as spatial navigation. It is also densely packed with neurons and therefore proves to be a good model for neurophysiology studies. In a study that performed neuronal analysis on two children suffering from autism, it was

found that their hippocampal neurons were smaller in area and dendritic branching than age-matched controls (Raymond *et al*., 1996). This supports the idea that children suffering from autism have slowed maturation of neurons and that the hippocampus may play an important role in autistic behaviors.

RNA extraction

In order to analyze if the siRNA was successful at silencing the targeted gene in the area of injection, RNA extraction post-mortem is necessary. RNA extraction is commonly accomplished using reverse transcriptase kits provided by the company *Qiagen*. For the mRNA extraction, the Qiagen's RNAeasy kit (Qiagen, Valencia, CA) has proven to be effective with many different tissues including brain matter, saliva, and buccal cells. It contains relatively easy steps and materials that allow for sufficient mRNA extraction before PCR (Spivack *et al*., 2004). Once RNA has been successfully extracted from the tissue sample, real-time PCR can be performed with known primers (Ball *et al*., 2002).

Behavior testing

Object recognition tests have been performed on mice to assess changes in memory function. A normal mouse, when faced with a familiar and a novel object, should spend more time with the novel object (Figure 4), since mice are naturally curious. The mouse's attention to the novel object will usually consist of sniffing and approaching the object from different angles. Studies involving a mouse with lowered memory capabilities have shown that the mouse with

lowered memory function, when faced with a familiar and novel object, will not remember the familiar object and will therefore treat both objects as novel (Reibaud *et al*., 1999). Tests have also been done involving the enhancement of a mouse's memory capabilities. Object recognition testing with memory enhanced mice showed greater ability of those mice to remember the familiar object for longer periods of time, and therefore show increased attention to the novel object over that longer time span (Reibaud *et al*., 1999).

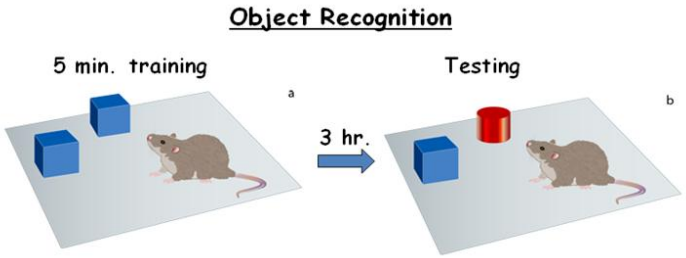

Figure 4: Object Recognition test

To test the anxiety of a mouse, an open field test can be administered. In an enclosed area, a normal mouse will stay close to the walls for a while, then become curious and venture out into the middle of the open field. However, an anxious mouse will remain almost exclusively at the walls of the enclosure (Figure 5). In one study, high-anxiety mice were created by using a targeting vector to limit corticotrophin-releasing hormones. Their anxiety was confirmed using an open field test (Bale *et al*., 2000).

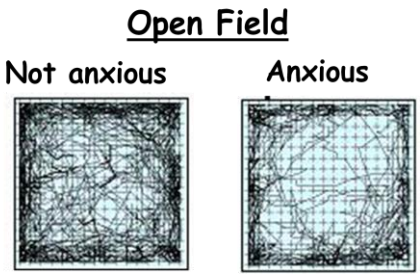

Figure 5: Open field test for Anxiety

Previous research in this lab

Previous research in this lab has been conducted involving Jarid1c and its effect on behavior when knocked down in the hippocampus. During a study performed by Marc Siegel, Liz Byrnes, Ph.D, and Jun Xu, Ph.D, Jarid1c was knocked down using gene specific siRNA. Four female C57 BL/6 mice were injected bilaterally with Jarid1c-siRNA using stereotaxic injection into their hippocampus. Four additional mice were injected with a non-targeting control siRNA. The mice's behavior was then tested two days post-surgery to allow for the siRNA to take full effect, since two to four days post-surgery is optimal for testing. Behavior tests were performed for social preference, aggression, and object recognition. Social preference tests the mouse's time spent investigating a novel mouse, aggression tests the mouse's aggression towards the novel mouse and object recognition tests memory as previously described.

Since this study used female mice, it was important to test their estrogen levels. Estrogen levels have been shown to affect behavior, so estrogen levels must be assessed when conducting behavior testing on female mice (Fernandez *et al*., 2008). Therefore, estrogen levels were recorded using vaginal smearing after the behavior tests for both the test mice and the control

mice. The mice were then categorized into one of two groups, high or low estrogen, depending upon their estrogen levels.

The success of the Jarid1c knockdown was quantified using the RT-PCR method on a real-time PCR machine (ABI, Foster City, CA). It was found that the Jarid1c-siRNA samples had a 60% reduction relative to the control samples. This quantitative analysis confirmed the effectiveness of the treatment.

During analysis of the behavior testing, it was found that for both high and low estrogen mice, the Jarid1c-siRNA mice spent less time with the stimulus mouse than the controls during the social preference test ($p < .01$). During the object recognition tests it was found that Jarid1c-siRNA mice spent less time with the novel object than the control mice ($p=0.09$). However, it was also noticed that the level of estrogen affected the performance of the control mice in the object recognition test. Those with higher estrogen levels performed better than those with lower estrogen levels. This was not seen in the Jarid1c-siRNA treated mice or in any other behavior test. It was also found that there was no change in aggression between Jarid1c- and control-siRNA treated mice.

In conclusion, this previous study found that in female BL/6 mice, knockdown of Jarid1c in the hippocampus caused a lowered desire to socialize. This was displayed by the results in the social preference test. Knockdown of Jarid1c in the hippocampus also affected the mouse's memory as shown by the object recognition test. However, the object recognition test also found that estrogen levels affect behavior during the testing process for the control mice but not the Jarid1c-siRNA treated mice. Lastly, it proved that the use of stereotaxic injection to perform gene specific siRNA knockdown in the hippocampus of these mice was successful. To further

the investigation into Jarid1c and its ties to XLMR symptoms, this study looks into the knockdown affects of Jarid1c in three areas of the mouse brain, the hippocampus, prefrontal cortex and amygdala, measured using behavior testing. To date, little is known about the role of Jarid1c in different areas of the brain. The three brain regions chosen for investigation in this study were selected based on their previously discovered links to autistic and other XLMR symptoms in humans. This study hopes to answer questions about the importance of Jarid1c and whether XLMR symptoms may be caused by a deficiency of Jarid1c in these specific brain regions.

Materials and Methods

Animals

Male BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME) at 9 weeks of age. Each mouse was injected with an AVID chip (Norco, CA) a week after arrival at Tufts University to allow for easy identification using the AVID chip reader. Mice were kept on a 12/12 hour light/dark cycle with water and food available at all times. Mice also received daily attention from the research staff at Tufts University. All protocols involving mice were approved by the Tufts University Institutional Animal Care and Use Committee.

RNA interference

Jarid1c-siRNA was obtained from Qiagen (Valencia, CA). Jarid1c-siRNA contains four different siRNA sequences that each targets a different site on the Jarid1c mRNA. This broad targeting ensures a marked gene knockdown. Target sequences are:

Jarid1c-3: CAGCTTCGTCGTACCAACTCA

Jarid1c-6: ACGGCGGATCTTGGACCTCTA

Jarid1c-1: CGCATTGTTTATCCCTATGAA

Jarid1c-2: AAGATTGTTGGTGTGTAATTA

Upon arrival at Tufts University, the siRNA was resuspended in water (7.5 μg /1 μl) and mixed. On the day of surgery 3μl of siRNA suspension was mixed with 3μl of DOTAP, a transfection reagent (Roche, Indianapolis, IN). The same process was done with the control-siRNA which is non-targeting.

Stereotaxic siRNA delivery. (Detailed protocol is available in Appendix A).

Four mice were given siRNA injections each surgery day. Two brain regions were chosen on each surgery day and for each brain region one mouse received the Jarid1c-siRNA and the second received the control-siRNA. For example, on day 1 of surgery one mouse received a Jarid1c-siRNA injection in the prefrontal cortex, and one received a control-siRNA injection in the prefrontal cortex. Then another two mice received injections following the same procedure in the amygdala. The injections were given bilaterally with a concentration of 3.75 μg of siRNA in 1 μl. Over 7 surgery days, a total of 26 mice were given injections in the prefrontal cortex, hippocampus and amygdala (see Appendix B). Coordinates for each region are shown in Table 1.

In mm relative to Bregma (Paxinos and Franklin, 2001)	AP	ML	VD
Prefrontal cortex	+2.8	+/- 1.6	-1.3
Hippocampus	-2.0	+/-2.0	-1.6
Amygdala	-1.5	+/-2.6	-5.7

Table 1: Coordinates for targeted brain regions

Isoflourine was used for general anesthesia purposes as well as bupivicaine as a local anesthetic. Ketoprofen was given as a pain reducer. Sterile technique was used throughout the surgery and post-surgery monitoring was performed until full recovery.

Behavior testing (*written by contributing author, Alex Newbury, from the College of the Holy Cross)*

Two days after the siRNA injection, each mouse was tested in three different behavioral paradigms: anxiety and activity monitoring, and object recognition. Each test was conducted in the same behavioral testing room under relatively constant conditions (i.e. ~70^{o}F, 40% humidity). Every mouse was first habituated to the room for thirty minutes by being placed in our activity monitor. This apparatus quantifies the amount of locomotion for each mouse in thirty minutes. Once compiled, this data allows us to ensure that any lack of social or novel object interaction is in fact due to a disinterest in our stimulus and not due to any physical motor failures.

In addition, using the activity software *Motor Monitor*, we can gauge the amount of anxiety in each mouse by looking at the ratio of time spent in the two zones of the testing cage. Zone one is located around the perimeter, while zone two is located in the center. Any time spent in zone two is considered anxiety provoking, while time spent in zone one is considered to provide an anxiolytic effect. Subtracting the time spent in zone two from zone one allows us to see how often each mouse engaged in the anxiety provoking behavior, which corresponds to a greater amount of time in the center of the cage vs. the perimeter.

Once these tests were conducted, object recognition testing was performed. This behavioral paradigm must be conducted in two phases, as it tests the cognition and recall ability of each test mouse. Phase one consists of introducing each mouse to two identical objects for five minutes in a 2x2’ enclosure. In this study, two orange or blue toy cylinders were used at this stage. After a consistent latency of two hours and 45 minutes (165 minutes), each mouse was reintroduced to the same testing enclosure with one of the same objects from phase one and

another completely novel object. In this study, we used orange and blue pyramids. We scored the behavior by timing how long it took for the subject to spend thirty seconds with the novel object. We did this by running two stopwatches, one for the overall time and one for every time the subject was interacting with the novel object. Once thirty seconds was reached, the overall timer was stopped, the time was recorded and we returned the subject to its home cage. After each trial, both the testing apparatus and the toys were washed with 70% ethanol and then with water to remove the EtOH.

Microarray analysis

Two days post surgery, after behavior testing was completed, mice were decapitated and their brains collected. Brains were then preserved and frozen at -80 degrees Celsius. Once all of the surgeries and behavior testing was completed, brain samples were collected from the frozen mouse brains. The targeted region for each injection was located and a sample was collected using a 0.5mm micro-puncher while the brain was mounted on a cryostat. Tissues were pooled based on location and type of injection (Jarid1c-siRNA/control-siRNA). Two tubes for each group were used and samples were taken from each side of the brain. Samples were placed in the two tubes randomly to avoid any laterality effect.

After all samples were collected, RNA was extracted using an RNAeasy kit (Qiagen, Valencia, CA). RNA samples were then sent to the Computational Genomics Core at Tufts University Center for Neuroscience Research to be analyzed for gene knockdown success. The genome-wide expression profile was determined using the GeneChip Mouse Gene 1.0 ST Array (Affymetrix, Santa Clara, CA). To ensure accurate measurement of gene expression, 27 probes were used to represent all 28,853 genes.

Results

Brain injection with Jarid1c-siRNA results in knockdown of Jarid1c gene expression

To test the success of Jarid1c knockdown in the targeted areas, expression was quantified using the RT-PCR method. Samples from each brain region were analyzed on a real time PCR machine (ABI, Foster City, CA) and it was found that Jarid1c-siRNA treated samples showed a 25% reduction of Jarid1c activity relative to control samples. This analysis confirms successful siRNA treatment.

Object recognition was affected by Jarid1c knockdown in the hippocampus

In the object recognition test, mice with Jarid1c knockdown in the hippocampus spent less time with the novel object than the control mice (p=.014). This shows a significant memory deficit connected with a decrease in Jarid1c activity in the hippocampus (Figure 6).

In the prefrontal cortex and amygdala there was no significant difference in object recognition between those mice that received Jarid1c-siRNA or control-siRNA

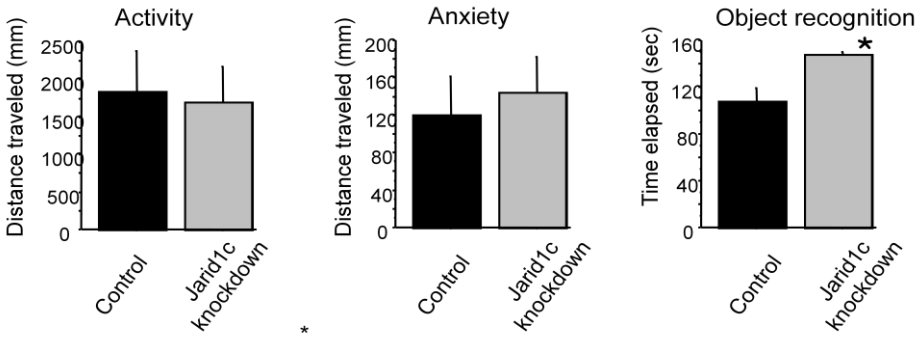

Figure 6: Jarid1c knockdown in the hippocampus

Anxiety was affected by Jarid1c knockdown in the prefrontal cortex

Anxiety was monitored for each mouse using the open field test. It was found that those mice with Jarid1c knockdown in the prefrontal cortex were significantly more anxious than the controls (p=.013). This test suggests that the Jarid1c gene in the prefrontal cortex has a relation to anxiety (Figure 7).

Knockdown of Jarid1c in the hippocampus and amygdala did not show any significant differences in anxiety levels between the knockdown mice and the controls

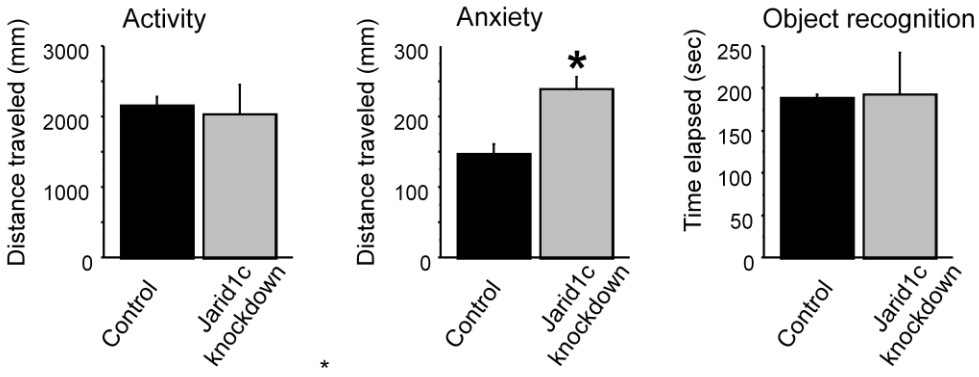

Figure 7: Jarid1c knockdown in the prefrontal cortex

Activity was not affected by Jarid1c knockdown

Activity was monitored for all of the mice tested in this study. It was found that when comparing the mice that received Jarid1c-siRNA with the mice that received the control- siRNA there was no significant difference in activity levels for any brain region (Figures 6 and 7).

Discussion

Using stereotaxic procedure to knock down Jarid1c activity in three different brain regions of the mouse yielded exciting results that significantly influence the study of X-linked mental retardation. Behaviors were observed in the Jarid1c knockdown mice that are indicative of similar behaviors seen in human XLMR patients. This makes the Jarid1c knockdown mouse an excellent model for XLMR studies.

Performing Jarid1c siRNA induced knockdown in the hippocampus showed a significant decrease in memory capability in the mice, as shown by the object recognition test. Jarid1c-siRNA mice took significantly longer to spend 30 seconds with the novel object than the control-siRNA mice. This shows a decreased ability to recognize the familiar object. Decreased cognition and memory disabilities are common symptoms of XLMR. The data strongly suggests that Jarid1c could have a role in causing these symptoms in humans.

siRNA knockdown of Jarid1c activity in the prefrontal cortex also led to behaviors similar to those seen in XLMR patients. The prefrontal cortex has been known to play important roles in social behavior in humans. Therefore, it was exciting to see that Jarid1c knockdown in the prefrontal cortex induced increased anxiety as shown in the open field test. Heightened anxiety levels are very prevalent in XLMR cases, especially autism. This mouse model shows a link between the role of Jarid1c in the prefrontal cortex and the common symptom of high anxiety levels in XLMR in humans.

No changes in activity were noticed between control and Jarid1c-siRNA treated mice. This is important because it supports the results of the anxiety test. If the activity levels were significantly different between the groups it could call into question results related to anxiety

levels. If activity levels had been affected, increased movement throughout the field in the open field test might have been due to hyperactivity and not lowered anxiety levels.

Using real-time PCR to determine the actual knockdown success is important for verifying results. If the knockdown was shown to be unsuccessful, then the behavior results could have been due to a lesion effect during stereotaxic injection, and not the siRNA. However, given the success of the knockdown (25%) the behavior results can be attributed to the success of the Jarid1c knockdown. It is important to note that while 25% may not seem like a significant knockdown, given the behavior results attributed to this knockdown it shows the importance of Jarid1c activity.

In addition to the hippocampus and the prefrontal cortex, the amygdala was also injected. However, there were no significant behavior results from this region. Possible reasons could be that Jarid1c does not play as important of a role in this region. On the other hand, it could have been due to location and complications with the stereotaxic procedure. The amygdala is extremely ventral in the brain, and because of this the injection needle must go through a lot of brain matter to reach the target area. Given the distance the needle must travel there is a higher likelihood that all mice, Jarid1c-siRNA and control-siRNA, could be affected by the procedure to the degree that any differences in behavior due to Jarid1c knockdown would be undetectable.

The results of this experiment provide a strong argument for the involvement of Jarid1c in XLMR, particularly in the brain regions of the hippocampus and prefrontal cortex. Further studies should pursue the pathway involving Jarid1c and investigate the genes that are down-regulated by it. Looking further into the pathway will help to pinpoint the actual cause of these behaviors. Further research should investigate whether it is the specific knockdown of Jarid1c

that is causing behavior changes, or if the knockdown of Jarid1c causes an inactivity of another part of the pathway which in turn causes these behavior changes.

Additional future research should investigate the long term behavior changes caused by silencing Jarid1c in the brain. If Jarid1c is silenced permanently using a lentivirus approach, resulting long term behavior changes could be monitored. Without this investigation it will remain unknown whether behavioral changes might worsen with time, or whether a compensation mechanism will develop, allowing conditions to improve. In addition, the knockdown effect on different ages of mice should be investigated.

This study helped support the hypothesis that Jarid1c mutations have a causal effect on XLMR. It was found in particular that knockdown of Jarid1c in the hippocampus and prefrontal cortex caused reduced feature recognition and increased anxiety, respectively. Microarray analysis indicated that knockdown was 25% successful relative to the non-targeting control-siRNA and that expression of Jarid1c was not affected in nearby striatal regions. Lastly, it was found that expression of Jarid1d, a close homologue of Jarid1c, was not affected by the knockdown procedure, which argues against an off-target effect.

It is hoped that these results and future experiments involving Jarid1c's involvement in XLMR will help to find the cause of these associated symptoms. Given these results and the results of future suggested studies, a cure for XLMR diseases will be attainable.

Literature Cited

1. Akaneya Y, Jiang B, Tsumoto T. RNAi-Induced Gene Silencing by Local Electroporation in Targeting Brain Region. J Neurophysiol. 2005; 93:594-602.
2. Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee KF. Mice deficient for cotricotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nature Genetics. 2000; 24:410-414.
3. Ball HJ, McParland B, Driussi C, Hunt NH. Isolating vessels from the mouse brain for gene expression analysis using laser capture microdissection. Brain Research Protocols. 2002; 9:206-213.
4. Baron-Cohen S, Ring HA, Bullmore ET, Wheelwright S, Ashwin C, Williams SCR. The amygdala theory of autism. Neuroscience & Biobehavioral Reviews. 2000; 24:355-364.
5. Cetin A, Komai S, Eliava M, Seeburg PH, Osten P. Stereotaxic gene delivery in the rodent brain. Nature Protocols. 2007; 1:3166-3173.
6. Chelly, J. Breakthroughs in molecular and cellular mechanisms underlying X-linked mental retardation. Human Molecular Genetics. 1999; 8:1833-1838.
7. Fernandez SM, Lewis MC, Pechenino AS, Harburger LL, Orr PT, Gresack JE, Schafe GE, Frick KM. Estradiol-induced enhancement of object memory consolidation involves hippocampal extracellular signal-regulated kinase activation and membrane-bound estrogen receptors. 2008; 28(35): 8660-7.
8. Iwase S, Lan F, Bayliss P, de la Torre-Ubieta L, Huarte M, Qi HH, Whetstine JR, Bonni A, Roberts TM, Shi Y. The X-Linked Mental Retardation Gene SMCX/JARID1C Defines a Family of Histone H3 Lysine 4 Demethylases. Cell. 2007;128:1077–1088.
9. Jensen LR, Amende M, Gurok U, Moser B, Gimmel V, *et al*. Mutations in the JARID1C Gene, Which Is Involved in Transcriptional Regulation and Chromatin Remodeling, Cause X-Linked Mental Retardation. The American Journal of Human Genetics. 2005; 76:227–236.
10. McCaffrey AP, Meuse L, Pham TTT, Conklin DS, Hannon GJ, Kay MA. Gene expression: RNA interference in adult mice. Nature. 2002; 418:38-39.
11. Ohnishi T, Matsuda H, Hashimoto T, Kunihiro T, Nishikawa M, Uema T, Sasaki M. Abnormal regional cerebral blood flow in childhood autism. Brain. 2000; 123:1838-1844.
12. Paxinos G, Franklin KBJ. The Mouse Brain in Stereotaxic Coordinates. Academic Press. 2001; 2 ed.
13. Raymond GV, Bauman ML, Kemper TL. Hippocampus in autism: a Golgi analysis. Acta Neuropathol. 1996; 91:117-119.
14. Reibaud M, Obinu MC, Ledent C, Parmentier M, Bohme GA, Imperato A. Enhancement of memory in cannabinoid CB1 receptor knock-out mice. European Jrnl of Pharm. 1999; 379:R1-R2.

15. Santos C, Rodriguez-Revenga L, Madrigal I, Badenas C, Pineda M, Mila M. A novel mutation in JARID1C gene associated with mental retardation. Eur J Hum Genet. 2006; 14:583–6.
16. Spivack SD, Hurteau GJ, Jain R, Kumar SV, Aldous KM, Gierthy JF, Kaminsky LS. Gene-Environment Interaction Signatures by Quantitative mRNA Profiling in Exfoliated Buccal Mucosal Cells. Cancer Research. 2004; 64:6805-6813.
17. Thakker DR, Natt F, Huskin D, Maier R, Muller M, van der Putten H, Hoyer D, Cryan JF. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. PNAS. 2004; 101:17270-17275.

Appendix A: Stereotaxic injection procedure

1. Autoclave surgery tools: scalpel blade holder, forceps, staple administrator, staples, and cotton swabs.
2. Day of surgery: set up heating pad on surgery table and place mice cage half on to begin acclimation.
3. Wipe down all surgery areas with 70% ethanol.
4. Turn on bead sterilizer.
5. Lay down chux pads on the surgery area and preparation area.
6. Place stereotaxic instrument on chux pad on surgery table and wipe down with 70% ethanol.
7. Set up preparation area with a gram scale, the induction chamber, nose cone, cotton swabs and disinfecting solutions (surgical scrub, 70% ethanol, and surgical paste).
8. Lay out a drape next to the surgical instrument using sterile techniques. Open the surgical pack and dump the instruments into the sterile field. Also dump sterile cotton swabs, and a 10 blade onto the drape. Finally, lay a pack of sterile gloves next to the drape.
9. Put on hair net, face mask and non-sterile gloves.
10. Load a 1µL syringe with injectable (Jarid1c-siRNA or control siRNA) after wiping down with 70% ethanol and then sterilizing it via the bead sterilizer. Then load into the stereotaxic instrument.
11. Once the mice have been in the room for at least 10 minutes to acclimate, remove first mouse, place in induction chamber and turn on isoflourine delivering at 4% for about 2-4 minutes or until the animal's respiratory rate has slowed significantly. Make sure oxygen is set at around 1 and exhaust is set a little above 1.
12. When the mouse's breathing rate has decreased a significant amount, turn on the isoflourine to the nose cone on the preparation table, reducing the flow to 2-2.5% and turn off the flow to the induction chamber. Move mouse from induction chamber to nose cone immediately after.
13. Perform a toe press to make sure the mouse is fully under.
14. Weigh the animal and record weight once you get no response from the toe press test. Once weight is gathered immediately place back on nose cone.
15. Begin pulling hair from top of the head until it is clear from ear to ear and from the eye line to the base of the skull.
16. Begin scrubbing. Start with the surgical scrub, then 70% ethanol applied with the cotton swabs and repeat 3 times. End with the surgical paste. Begin scrubbing at the center of the hairless area moving outward in a circular motion.
17. When scrubbing is complete, turn on isoflourine into the nose cone on the stereotaxic instrument. Turn off flow to nose cone in preparation area.
18. Move mouse to surgical instrument. Make sure nose is in cone and set incisors into hole on bar below nose cone to assist with stability.

19. Move the two ear bars so they are snug against the side of the mouse head to ensure stability and keep the head parallel to the table. Bars should be placed right at the ears and secured tightly while making sure that the pressure is light but secure. Test stability by pressing the top of the head with a sterile cotton swab.
20. Take off non-sterile gloves and put on sterile gloves using the proper sterile technique.
21. Give around 0.02CC of bupivicaine, a local anesthetic, subcutaneously at the incision site.
22. Make a 3-4cm midline incision on the top of the scalp.
23. Expose the skull and scrap skull lightly with scalpel. Then, wipe with a sterile cotton swab.
24. Locate the bregma and move the syringe so that it is just touching the bregma. Zero the x, y and z coordinates of the injection machine.
25. Move the syringe to the coordinates for the targeted injection site:
    a. Hippocampus - Lateral +2/-2 mm, Posterior -2.0 mm, and Ventral -1.6 mm
    b. Prefrontal Cortex – Lateral +1.6/-1.6 mm, Anterior 2.8 mm, and Ventral -1.3 mm
    c. Amygdala – Lateral +2.6/-2.6 mm, Posterior -1.5 mm, and Ventral -5.7 mm
26. Note where the syringe is placed and move it up out of the way. At the spot where the syringe will enter drill a hole using a dental drill. While drilling double check placement by moving the syringe down.
27. Once through the skull, slowly move the syringe down to the ventral coordinate and begin injection.
28. Injection will last for 5 minutes. Once complete leave in place for an additional 2-5 minutes.
29. After the wait period, slowing remove needle and replace syringe with same injectable before moving to the other side.
30. Re-zero the coordinates at the bregma and move to the opposite side of the skull maintaining the same anterior/posterior and ventral coordinates.
31. Drill at the spot of injection and slowly insert needle.
32. Inject at the same 5 minute pace, and wait the 2-5 minutes before removing.
33. Once needle is fully removed, turn off isoflourine and oxygen and get out 2 clamps and clamp applicator.
34. Using tweezers at the base of the incision to hold incision together, place 2 clamps on incision to keep it closed and allow it to heal. If incision doesn't look fully closed, a small amount of tissue glue can be applied.
35. Record time that mouse was taken off of gas.
36. Give appropriate amount of Ketoprofen. Ketoprofen is to be given at a dose of 10mg/kg once diluted appropriately (see directions on Ketoprofen bottle). Give subcutaneously and record dose on surgery card.

37. Place mouse in a prepared, clean, recovery cage with a few food pellets on the ground and water available. Make sure recovery cage is placed half on a heating pad.
38. Watch the mouse during recovery and record any unusual behavior as well as time of full alertness. Record on surgery card the time when the mouse is viewed as BAR. Mouse should not take longer than one hour to be fully recovered.

If any problems arise during surgery or mouse does not fully recover within one hour, immediately call the PI or the DTRR veterinarian on duty

Appendix B: Surgery details

Mouse ID	Date of Surgery	Injection site	siRNA Injection
550 627	2/18/2009	Prefrontal Cortex	Control
562 887	2/18/2009	Prefrontal Cortex	Jarid1c
567 840	2/18/2009	Medial Amygdala	Jarid1c
581 085	2/18/2009	Medial Amygdala	Control
032 343	2/25/2009	Dorsal Hippocampus	Control
588 000	2/25/2009	Dorsal Hippocampus	Jarid1c
540 054	3/4/2009	Dorsal Hippocampus	Control
542 886	3/4/2009	Prefrontal Cortex	Jarid1c
571 088	3/4/2009	Prefrontal Cortex	Control
582 837	3/4/2009	Dorsal Hippocampus	Jarid1c
563 803	3/18/2009	Dorsal Hippocampus	Jarid1c
575 554	3/18/2009	Medial Amygdala	Control
581 094	3/18/2009	Dorsal Hippocampus	Control
581 328	3/18/2009	Medial Amygdala	Jarid1c
539 860	3/21/2009	Medial Amygdala	Jarid1c
552 828	3/21/2009	Medial Amygdala	Control
558 619	3/21/2009	Prefrontal Cortex	Jarid1c
577 564	3/21/2009	Prefrontal Cortex	Control
023 829	3/25/2009	Dorsal Hippocampus	Control
536 612	3/25/2009	Medial Amygdala	Jarid1c
544 295	3/25/2009	Medial Amygdala	Control
574 574	3/25/2009	Dorsal Hippocampus	Jarid1c
019 607	4/1/2009	Dorsal Hippocampus	Jarid1c
543 058	4/1/2009	Dorsal Hippocampus	Jarid1c
836 060	4/1/2009	Prefrontal Cortex	Jarid1c
No ID	4/1/2009	Prefrontal Cortex	Jarid1c